Growth phase dependent stop codon readthrough and shift of translation reading frame in *Escherichia coli*

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Abstract Nonsense codon readthrough and changed translational reading frame were measured in different growth phases in *E. coli*. The strains used carry plasmid constructs with a translation assay reporter gene. This reporter gene contains an internal stop codon or a run of U-residues. Termination or frameshifting give rise to stable proteins that can be physically quantified on gels along with the complete protein products. Readthrough of the stop codon UGA by a nearcognate tRNA is several fold higher in active growth than in late exponential phase. In early exponential phase, about 7% of −1 frameshift at a U_9 slippery sequence is detectable; upon entry to stationary phase this frameshifting increases to about 40% followed by a decrease in stationary phase. A similar increase is observed in the case of +1 reading frameshift at the U_9 sequence, which increases from 13% in early exponential growth phase up to 38% at the beginning of stationary phase followed by a decrease. Thus, the levels of both stop codon readthrough and frameshifting are growth phase dependent, though not in an identical fashion.

Key words: UGA readthrough; Poly-uridine sequence; Frameshift; Growth phase; *Escherichia coli*

1. Introduction

The translation process normally results in 10^{-3}–10^{-4} mistakes per amino acid residue incorporated into the protein product [1]. Apart from such stoichastic errors, some genes include signals that give programmed error rates between 1% and 100%. The purpose of programmed errors, where one gene results in two products by frameshifting or stop codon readthrough, is probably to ensure production of a low but significant amount of a second protein that is encoded in the mRNA. There are many examples of programmed translational errors in eukaryotic viruses, both stop codon readthrough [2] and frameshifting at shifty sequences in retroviruses [3] and in the Ty retrotransposon in yeast [4]. The expression of the mammalian protein antizyme, which is important for polyamine biosynthesis, is auto-regulated by polyamines that modulate the frameshifting required to translate the antizyme gene [5].

In *E. coli* one remarkable example of programmed stop codon readthrough is provided by the gene *fdhF*, which carries an internal UGA codon that functions as a sense codon for the incorporation of selenocysteine [6]. High level programmed −1 frameshifting has been reported for *dnaX*, which codes for two subunits of DNA polymerase III [7–9]. Several transposase genes in *E. coli* use programmed frameshifting [10–12]. High level +1 frameshifting at an internal UGA codon has been found for the *E. coli* gene *prfB*, which codes for protein release factor 2 (RF2) [13]. In this gene the frameshifting is enhanced by a short Shine-Dalgarno like sequence, upstream of the shift site. This sequence base pairs with the 16S rRNA anti-Shine-Dalgarno sequence [14]. The frameshifting in RF2 is counteracted by an increased level of RF2 [5], probably due to a successful competition with the frameshift promoting tRNA.

Translational frameshifting and readthrough have been studied, in most cases, in bacterial cells in vivo during exponential growth. However, for bacteria in natural environments, exponential growth is rather rare [15] and laboratory conditions seldom reflect the natural growth situation. Natural growth conditions can be simulated by allowing the culture to enter stationary growth phase. Frameshifting has been shown to increase at a site which allows for −1 frameshifting in *E. coli* cultures upon entry into the stationary phase [16,17]. *E. coli* cells in stationary phase are not dormant, they remain metabolically active even after many weeks of starvation [15]. Growth phase also has effects on the intracellular concentrations of tRNA [18] and translation termination factors [19].

A semi-synthetic assay gene (3A′) that codes for three identical A′ protein domains has been constructed so that a DNA linker with test sequences can be inserted between the second and third A′ coding region. In this work we have inserted a UGA stop codon to measure the efficiency of termination and a U_9 sequence to measure reading frame slippage. Changed translational frameshifting or readthrough can be quantified by measuring the molar amounts of 3A′ protein and 2A′ protein formed. Here we present results that both stop codon readthrough and +1 and −1 frameshifting at a run of U-residues are growth phase dependent.

2. Materials and methods

2.1. Materials

[^{35}S]dATP was purchased from Amersham. Restriction enzymes and T4 DNA ligase were from Promega or BioLabs and Sequenase from United States Biochemical Corporation. Deoxyoligonucleotides were made by Pharmacia-Upjohn. A Midget electrophoresis system (Pharmacia-Upjohn) was used for gel electrophoresis.

2.2. Bacterial strains and growth media

E. coli strains MC1061 and HB101 [20] were used for routine cloning experiments and as host strains for the plasmids. Translational in vivo assays were made in *E. coli* UY211, a suppressor-tRNA negative, rifampicin sensitive derivative of XAc [21]. Bacterial cultures were grown at 37°C in a defined M9-based minimal medium supplemented with glucose and all the amino acids at recommended concentrations

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Abbreviations: IPTG, isopropyl-β-D-thiogalacto-pyranoside

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[22]. Plasmid-carrying strains were stabilised by using 300 μg/ml of ampicillin. Cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

2.3. Construction of plasmids

Standard recombination techniques were employed [23] and all synthetic sequences cloned were sequenced using a DNA Sequenase® kit (United States Biochemical Corporation). The sequence primer ABP02, which is complementary to a unique sequence at the 3′-end of the 3A′ reporter gene, was used for sequencing [24]. A 'wizard miniprep' protocol (Promega) was used for plasmid preparation. A Geneclean Kit (Bio Rad 101) was used to purify restriction fragments on 1% agarose gels.

Plasmid pAB25 [25], which is a modified form of pEZZL$_1$ZT [26–28], carries a semisynthetic gene coding for three identical derivatives of the IgG-binding B-domain from protein A of *Staphylococcus aureus*. The pAB25 plasmid was constructed by cloning the 3A′ gene from pAB4 [24] into pAB22 [29].

All plasmid constructs used in this study are derived from pAB25. A linker containing unique restriction site between the second and third A′ coding regions was used for cloning of synthetic deoxyoligonucleotides to make variants or the 3A′ gene. These inserts carry a run of dA$_9$, giving a U$_9$ homopolymer in the mRNA for frameshift determination, or code for mRNA with an internal UGA stop codon for readthrough determination (Fig. 1). To measure +1 frameshift a derivative was made from the zero-frame construct, pUY46 (Fig. 1a), by digestion with *Bam*HI, using its unique site in the linker downstream of the slippery sequence. The sticky ends were filled up using Klenow enzyme (Kw) and religated to give plasmid pUY46Kw (Fig. 1b). To measure −1 frameshift a derivative was also made starting from pUY46. The sticky ends obtained from the *Bam*HI digestion were exposed to Mung Bean nuclease (MB), in this case, giving blunt ends that were ligated to give plasmid pUY46MB (Fig. 1c). The +1 frameshifting in pUY44Kw and the −1 frameshifting in pUY46MB give a full length 3A′ protein as the product. Zero-frame reading or +1 or −1 frameshifting (depending on the plasmid used) gives two 2A′ proteins of similar sizes that co-migrate on the gel in one band. Plasmids pSM9 and pSM11 which were used for readthrough determinations have been described before [30].

2.4. Purification and determination of proteins

Cultures (1 l) were inoculated in M9-medium supplemented with all amino acids [22], from overnight cultures in the same medium, and grown for a total of 45 h. Samples (10 ml) of the culture were transferred to pre-warmed E-flasks with IPTG for induction of the *trc* promoter. Either after half an hour or three hours of induction (readthrough or frameshift determinations, respectively) the cells were harvested, put on ice, and A_{540} was measured. The cells were pelleted by centrifugation and the pellet was frozen. The frozen pellet was defrosted, resuspended in 1 ml 10×TST (0.5 M Tris (pH 7.4), 1.5 M NaCl, 5% w/v Tween 20) and incubated at 90°C for 8 min for lysis. The supernatant obtained after centrifugation was applied to a column containing IgG-Sepharose 6 Fast Flow (Pharmacia-Upjohn) and the eluated 3A′ and 2A′ proteins were analysed by SDS-PAGE and CBB G-250 staining (Fig. 2). The protein bands were quantified by laser densitometry, as previously described [24].

3. Results

3.1. Test system for measurements of stop codon readthrough and reading frame shift

An in vivo assay system was used to measure frameshift at a U$_9$ sequence and readthrough of a UGA stop codon. The assay system is a semisynthetic gene coding for three identical protein A′ domains, referred to as 3A′ ([31] and references therein). The A′ domain corresponds essentially to the IgG-antibody-binding B-domain in protein A from *S. aureus*

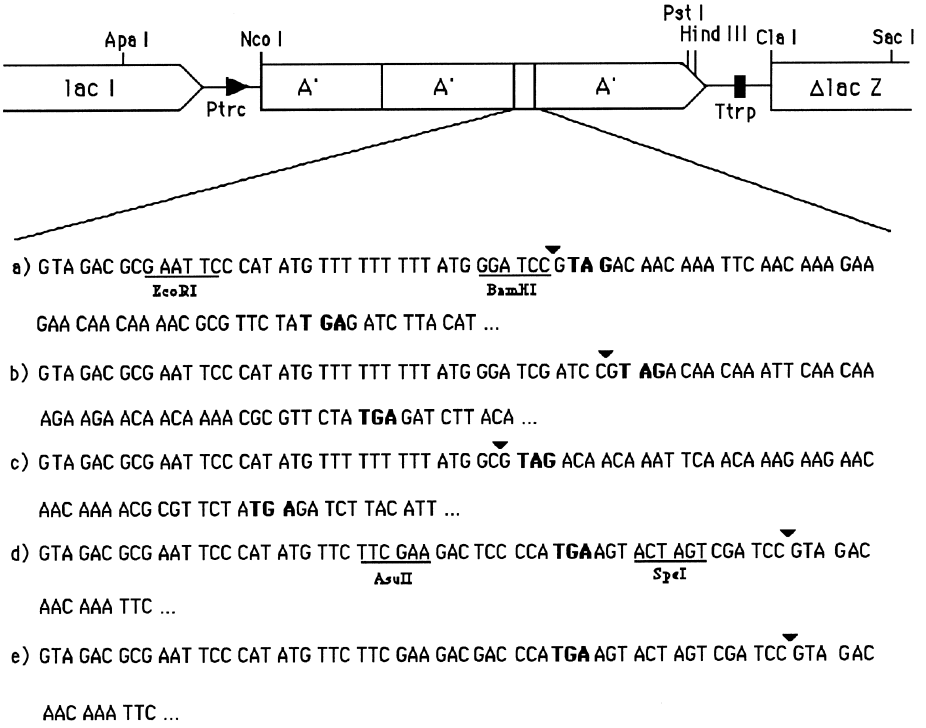

Fig. 1. The semi-synthetic 3A′ reporter gene. The 3A′ gene is transcribed from the P$_{trc}$ promoter, which is repressible by the *lacI* gene product, and transcription terminates at the T$_{trp}$ terminator. The first codon of the third A′ coding region is indicated by an arrow. a: pUY46; the zero frame T$_9$-construct in the linker between the second and third protein A domain is shown. Zero frame reading gives the full length 3A′ protein product. −1 and +1 frameshifting gives termination at indicated stop codons in bold. b: pUY46Kw; only +1 frameshifting gives the 3A′ protein. Zero frame reading and −1 frameshifting gives termination at indicated stop codons in bold. c: pUY46MB; only −1 frameshifting gives the 3A′ protein. Zero frame reading and +1 frameshifting gives termination at indicated stop codons in bold. d: pSM9; the linker between the second and third A− units contains a UGA stop codon with UCC two codons upstream (position −2) of the stop, giving low readthrough. e: pSM11; the linker contains a UGA stop codon and has a GAC codon at the −2 position, giving high readthrough [30].

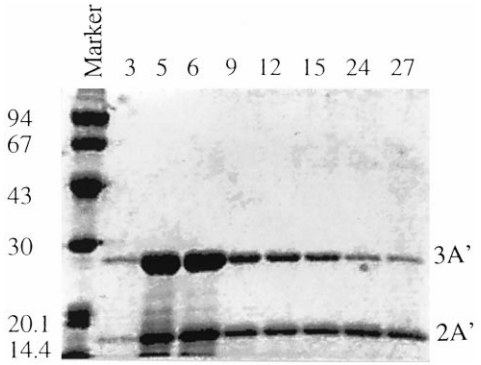

Fig. 2. Polyacrylamide gel electrophoresis of 3A′ and 2A′ proteins. Proteins in strain UY211 with pSM11 with a UGA stop codon in the linker between the second and third A′ coding domains were analyzed as described in Section 2. Samples were taken throughout the growth cycle and the times of harvest of the induced subcultures, after 30 min of induction with IPTG, are indicated. The 3A′ and 2A′ proteins and molecular weight (kD) of marker proteins are indicated.

[28,32]. This domain has earlier been referred to as domain Z and B, but is now denoted domain A′ in order to avoid confusion with other reporter genes. The assay gene is under the control of an inducible *trc* promoter, which is an IPTG-inducible variant of the *tac* promoter [33] and is repressed by the plasmid-coded *lac* repressor. The 3A′ gene variants used here lack the secretion signal coding sequence that precedes the first domain, as described in the original reports [24,28]. A DNA linker with test sequences can be inserted between the second and third A′ coding region. Changed translational frameshifting or readthrough can be quantified as an altered ratio between the molar amounts of 3A′ protein (24 kDa) and 2A′ protein (15 kDa) formed as revealed by gel electrophoresis.

For readthrough measurements, a UGA stop codon is introduced into the linker. Transmission values (T) are calculated using the molar amounts of 3A′ protein (readthrough product) in relation to 2A′ protein (termination product) produced (T = [3A′]/[2A′]). Two readthrough constructs were studied, plasmid pSM9 giving a relatively low readthrough because of a UCC codon two codons upstream of UGA (Fig. 1d), and plasmid pSM11 giving a higher readthrough because of a GAC codon at this position (Fig. 1e) [30,34]. The separation of the 2A′ and 3A′ proteins using polyacrylamide gel electrophoresis is illustrated in Fig. 2. There are no frameshifting promoting sequences in pSM9 and pSM11.

A 3A′ protein product originating from the pUY46Kw construct is the result of a +1 reading frameshift. This construct has one stop codon in the −1 reading frame at the beginning of the third A′ coding region and one stop codon in the zero-frame in the beginning of the same region (Fig. 1b). Both events give 2A′ proteins of similar length that comigrate on the gels; therefore only +1 frameshifting events give a full length 3A′ protein product. The pUY46MB construct is made in analogy with the Kw-construct, so that only the −1 frameshift gives a 3A′ protein product. This construct also has stop codons so that the zero frame or +1 frame-shift gives two similar 2A′ proteins (Fig. 1c) that do not separate from each other on gels. Frameshift frequency is determined using molar amounts of 2A′ and 3A′ proteins (fs = [3A′]/[[3A′]+[2A′]]). 2A′ thus represents two proteins of similar size resulting from termination at stop codons in the zero frame or from a −1 (pUY46Kw) or +1 (pUY46MB) frameshifting event.

3.2. Readthrough at different growth phases

Readthrough can be quantified as a transmission value (T = [3A′]/[2A′]). UGA readthrough was studied by following growth and protein production during 27 h. A strain with the plasmid pSM9 or pSM11, both carrying an internal UGA stop codon in the linker between the second and third A′ coding region, was used (Fig. 1d,e). Transcription from the *trc* promoter was induced by addition of IPTG to small subcultures half an hour prior to cell harvest. Fig. 3a and b show the transmission values in relation to growth phase. At stationary phase, readthrough is lower than during active growth. For cells containing pSM11, the level of readthrough is higher than for pSM9 due to the leaky character of the former stop codon context [30]. However, in both strains, a similar influence of growth phase can be seen. Similar results were obtained if the time of induction of the 3A′ gene was extended to three hours instead of half an hour (not shown). Similar results were also obtained for cultures that were started directly using a sample from an overnight culture as inoculum or that had been pre-grown to mid-log phase (Fig. 3, inserts).

3.3. Reading frame alterations at different growth phases

Fig. 4a and b show the levels of −1 and +1 frameshifting in relation to growth phase. The *trc* promoter was induced with IPTG three hours before the cells were harvested. Cells were lyzed and the 3A′ and 2A′ gene products were isolated using affinity chromatography on IgG-Sepharose ([31] and references therein). The relative molar amounts of the 3A′ and 2A′ proteins were measured after separation by gel electrophoresis. Growth and protein production were studied from lag phase through log phase and into stationary phase, for a total of 45 h. Samples were taken approximately every third hour.

It can be seen in Fig. 4 that in mid-exponential growth phase, frameshifting was at a relatively moderate level, but when the cultures reached the end of the log phase and entered the stationary phase, the frameshifting frequency increased. The −1 frameshifting value of about 6% in early exponential growth phase increases to more than 30% upon entry into stationary phase. A similar temporary increase in frameshifting is seen for +1 frameshifting, going from approximately 13% in early exponential growth phase to 38% in the beginning of the stationary phase. After the cultures have entered stationary phase, frameshifting decreases approximately down to the mid-log phase level. Thus, these experiments suggest that the increased −1 and +1 frameshifting seen when the cells are entering stationary phase is only transient.

4. Discussion

Stop codon readthrough is the result of competition between the release factor, giving termination, and a near-cognate tRNA, giving readthrough. In *E. coli* UGA readthrough is caused by $tRNA^{Trp}$ [24]. The most simple explanation for the growth-phase-dependent readthrough is a changed balance in the production of the near-cognate charged tRNA and/or the release factor(s), reflecting a change of cellular metabolism in the different growth phases [18,19]. However,

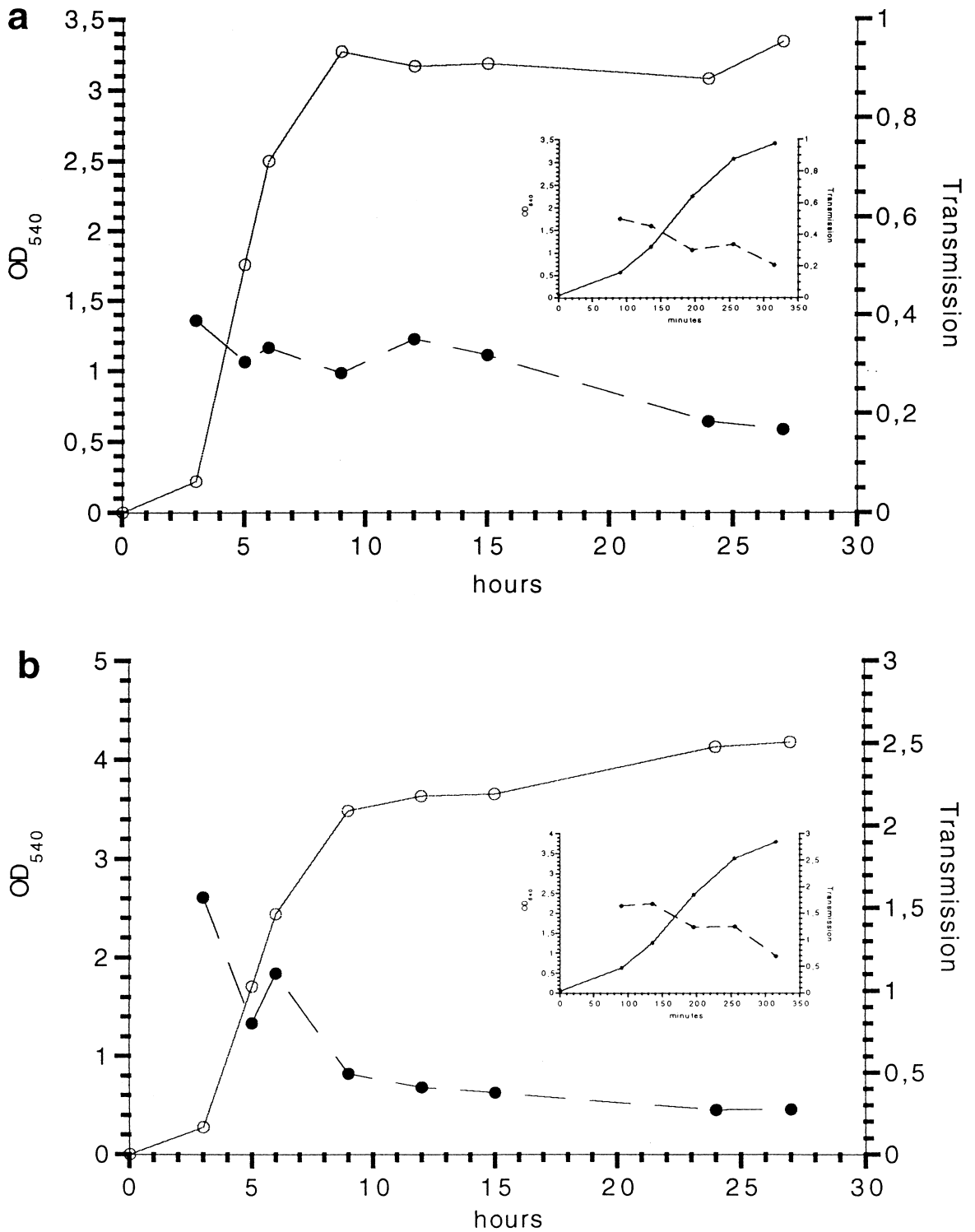

Fig. 3. Readthrough in different growth phases. Readthrough is given as transmission values (T = [3A′]/[2A′]). Transmission is shown with broken line and OD_{540} with solid line. The cultures were inoculated directly from overnight cultures (main figure) or from cultures that had been pre-grown to an OD_{540} of about 0.5 and then diluted in connection with inoculation (insert figure). a: Readthrough in pSM9. b: Readthrough in pSM11.

it is also possible that the translational machinery alters some of its properties with respect to error control during this phase.

Similarily, translational frameshifting can be affected by a change in expression of a tRNA that decodes in the shifted frame and that competes with the zero-frame-maintaining tRNA. The slippery sequence we analyzed has nine U-residues in the mRNA. In this case, transcriptional slippage also must be considered giving U_8 or U_{10} runs in the mRNA, since such slippage takes place at runs of A or T in DNA if the runs are long enough, up to 12 residues [35].

The changed frameshifting or nonsense codon readthrough

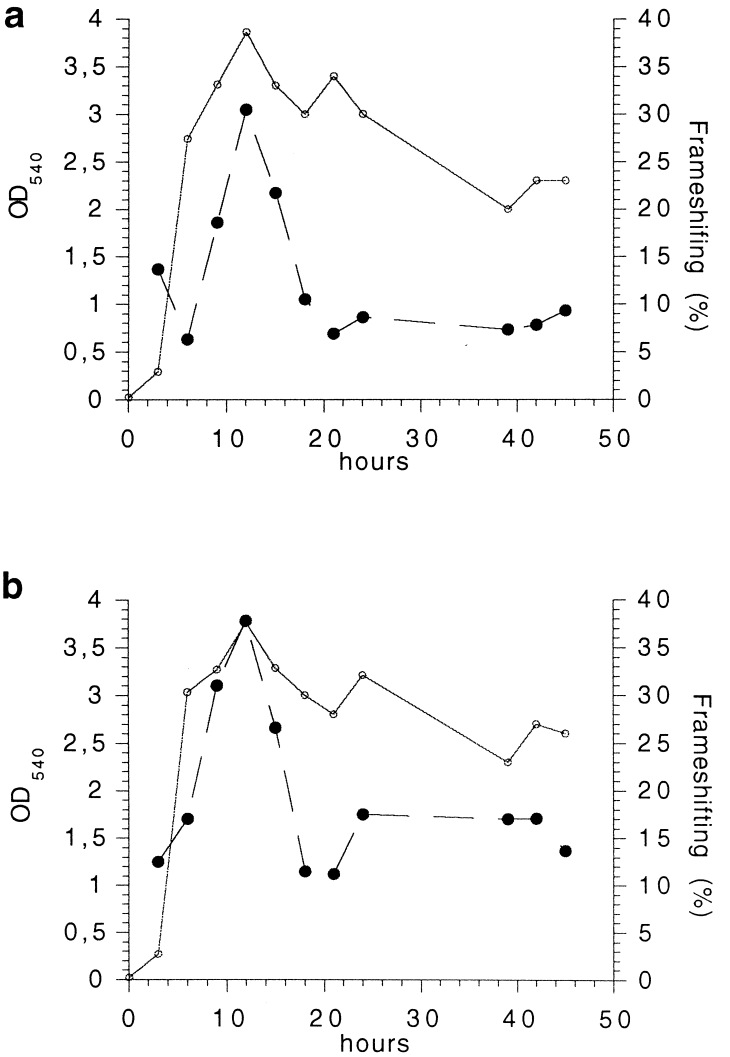

Fig. 4. Reading frame shifts (fs = [3A′]/[[3A′]+[2A′]]) in relation to growth phase. 2A′ denotes the two proteins that comigrate on gels and arise from zero frame and alternative frame termination. The broken line shows frameshifting, and OD_{540} is shown by the solid line. Samples were taken and subjected to IPTG induction (see Section 2) whereafter frameshifting and OD_{540} were determined, as shown. a: +1 frameshifting using pUY46Kw. b: −1 frameshifting using pUY46MB.

described here is observed by monitoring the gene products from both the zero frame and −1/+1 altered frame on gels. The 3A′ assay gene does not have any transcription polarity signals that could possibly influence the results [25]. We observe that readthrough is higher during active growth than it is in the stationary phase whereas for frameshifting there is a transient increase in connection with the transition to stationary phase. Translational frameshifting in *E. coli* has been reported to increase as cells enter stationary phase [16,17]. In this case, a −1 frameshifting-prone sequence, at the beginning of the *lac*-reporter gene was analyzed. The increasing frame-shifting observed in those experiments was extremely high, up to two orders of magnitude. The changed level of frameshifting we describe here using another shifty sequence is much lower, about 3–6 fold. Thus, the response of stop codon readthrough and shifted reading frame to growth phase are quite different in the indicated cases. This suggests a multitude of reasons for the appearance of such translational errors.

It is known that the transition period between exponential and stationary phase of growth involves drastic changes in both morphological and physiological cell properties; the general translation rate decreases to approximately 20% of the translational rate observed for cells in exponential growth [36]. The cultures analyzed here have been induced with IPTG for half an hour or three hours. This gives time for a reasonable physiological adaptation to the induced 3A′ gene and also for accumulation of 2A′ and 3A′ gene products for physical determinations on gels. Our cultures have been grown using a defined medium supplemented with amino acids and it is not known to what extent the exponential growth is limited by the different components in the medium, such as carbon source, aeration and pH. Slowly growing bacteria have a high pool of ppGpp [37]. The gene product of *rpoS*, the specific stationary phase sigma factor, induces expression of many genes and is positively regulated by ppGpp (for a review see [15]). ppGpp does not seem to have any direct effect on translational accuracy or on kinetics of translation in vivo. Instead, it probably acts at the level of transcription [38,39]. The strain used here is *relA*$^+$, meaning it has a normal capacity to produce ppGpp as a response to entering stationary phase. Further investigation is required to find out to what extent the apparently increased reading frameshift

and nonsense codon readthrough upon transition to stationary phase results from changed gene expression [18,19] or from changed properties of the translational machinery.

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